UNITED STATES PATENT APPLICATION

OF

JAN-HEINER KUPPER

ALEXANDER BURKLE

LEON VAN GOOL

AND

HARALD ZUR HAUSEN

FOR

MAMMAL WITH INHIBITION OF THE POLY (ADP RIBOSE) POLYMERASE AND METHOD FOR USING SAME TO IDENTIFY CANCERIGENIC AGENTS

INTELLECTUAL PROPERTY/TECHNOLOGY LAW
P.O. Box 14329 • Research Triangle Park, NC 27709

09/446808
430 Rec'd PCT/PTO 23 DEC 1999

K 2564

MAMMAL WITH INHIBITION OF THE POLY(ADP RIBOSE) POLYMERASE AND METHOD FOR USING SAME TO IDENTIFY CANCERIGENIC AGENTS

~~A Method for Identifying Cancerigenic Agents Using a Mammal With Inhibition of the Poly(ADP-Ribose)Polymerase~~

The present invention relates to a process for identifying cancerogenic agents.

A plurality of formerly unknown substances having cancerogenic danger potential and increasing in number because of constant new developments, occur in foodstuffs, cosmetics, textiles, materials, chemicals as well as other artificial products, but also in nature. In addition, physical agents (e.g. X-rays, U.V. radiation) can also cause cancer. Various in vivo and in vitro tests have been carried out so far to identify these substances and evaluate their cancerogenic potential.

The wide-spread Ames' test, which is also referred to as Salmonella typhimurium test, is based on the mutagenicity of substances in bacteria (Clonfero et al., Med. Lav. 81, pp. 3-10 (1990)). A likewise known in vitro test is the SOS chromotest (Quillardet et al., Mutat. Res. 297, pp. 235-279 (1993)) which is based on the induction of the bacterial SOS system by genotoxic agents. Both tests are comparable as regards their sensitivity but include the fundamental drawback that the genotoxic effects of substances in bacteria and higher organisms may differ and thus the results cannot be applied to the mammalian organism. Therefore the micronucleus test (Miller et al., Environ. Mol. Mutagen, 26, pp. 240-247 (1995), the single-cell gel test (SCG test, also referred to as cometary assay) and the test for sister chromosome exchange (Hartmann et al., Mutat. Res. 346, pp. 49-56 (1995) were developed few years ago. They are all based on eukaryotic cell systems.

For investigating the mutagenic effect of substances or physical agents in living organisms (in vivo), assays were developed which are based on the mutation of bacterial reporter genes (LacI or lac Z gene) which were inserted as transgene in mice (Gossen et al.,

Mutat. Res. 307, pp. 451-459 (1994). As a result, what is called the muta mouse and the big blue mouse were created.

Since the development of tumors is a process which comprises several factors and has not yet been elucidated in every detail, processes which only analyze individual aspects (e.g. mutation generation) of the tumor formation are insufficient. Therefore, the carcinogenicity of a substance or physical agent cannot be ruled out a priori in the case of a negative result. Furthermore, the bacterial systems usually used for the genotoxicity detection of chemical and physical agents can only be applied in limited fashion to higher organisms. Therefore, it must be stated that according to the current state of the art only the tumor formation as such is a safe parameter to determine the cancerogenic danger potential of a chemical or physical agent. For this reason, direct carcinogenicity tests using rodents had been introduced many years ago. However, in connection with these tests prescribed for the approval of new substances in many countries it is often necessary to apply very high doses of the corresponding substance so as not to obtain false negative results. Thus, the relevant objection was raised to the effect that the obtained positive results are not triggered by a real carcinogenicity of the substances but that only a non-specific stimulation of cell division is given which is due to overdosage. Mutations and, as a result, cancer would only form because of this mitogenic activity, so that these test which provide false positive results do not yield reliable results on the carcinogenicity of substances.

Therefore, it is the object of the present invention to provide a process by means of which it is possible to reliably identify cancerogenic agents.

This object is achieved by the subject matters defined in the claims.

The process according to the invention is carried out using a mammal, preferably a rodent, particularly preferably using a mouse, with DNA repair disturbance. The DNA repair disturbance is based on the trans-dominant inhibition of poly(ADP ribose)polymerase (abbreviated as PARP), which is an enzyme

involved in DNA repair processes. The inhibition of PARP is preferably based on the expression of a dominant negative mutant of poly(ADP ribose)polymerase, preferably the transgenic expression of such a mutant in a mammal, so that a transgenic animal is produced which is also a subject matter of the present invention. PARP has a DNA binding domain (abbreviated as DBD) which enables the binding to DNA strand breaks and results in an enzyme activity of PARP so as to enable repair of the strand breaks. However, a deletion is present in the dominant negative PARP mutant, so that only the DNA binding domain of PARP is expressed. This effects an inhibition of the PARP enzyme function and thus the DNA repair. The expression of this PARP mutant has no influence on the cell division and cell vitality in the absence of genotoxic stress. However, if genotoxic (chemical or physical) agents are applied, the PARP inhibition will lead to a considerable increase in the sensitivity of the cells to these treatments. The presence of the PARP mutant then results in an increased genetic instability after cancerogen treatment, which is manifested in an increased recombination as well as intensified gene amplification. Furthermore, the disturbance of the PARP function results in an increased mutagenicity of genotoxic agents. Thus, the disturbance of the cellular PARP function results in an increased rate of various genetic changes (mutations, recombinations, gene amplification) upon treatment with a cancerogen. As a function of the nature of the cancerogenic agent and the kind of its application, these various genetic changes permit various ways of tumor formation (e.g. oncogene amplification, tumor suppressor gene mutation or combinations thereof).

The mammal, preferably the transgenic mouse, used in the process according to the invention, has advantageously a skin-specific expression of the dominant-negative PARP mutant, every other organ-specific expression being, of course, also possible. However, because of its good accessibility and controllability the skin is the preferred organ for examinations as to carcinogenesis. For controlling the transgene it is possible to use every promoter known to the person skilled in the art and permitting a tissue-specific expression, preferably in the skin. The cytokeratin-14 promoter is used preferably. It permits an expression in the basal cell layer which is active as regards cell division and out of

which skin tumors develop preferably (Vassar et al., Proc. Natl. Acad. Sci USA 86, pp.1563-1567 (1989)).

It is preferred to use, for the production of the transgenic mammal, a fragment which has the following arrangement (see figure 1):

- 1.946 kb AvaI fragment of the human cytokeratin promoter (Vassar et al., Proc. Natl. Acad. Sci. USA 86, pp. 1563-1567 (1989))
- 1.156 kb DBD fragment from position -29 up to the internal NlaIV site at 1127 of the human poly(ADP ribose)polymerase (Cherney et al., Proc. Natl. Acad. Sci. USA 84, pp. 8370-8374 (1987))
- 0.486 kb of the polyadenylation signal of the human cytokeratin promoter (Vassar et al., Proc. Natl. Acad. Sci. USA 86, pp. 1563-1567 (1989))

A vector containing this fragment (pKDinoDBD) was deposited with the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen [German-type collection of microorganisms and cell cultures], Braunschweig) under number DSM 11594 on June 11, 1997.

The transgenic mammal is produced according to the method generally described by Hogan et al. ("Manipulating the mouse embryo: A laboratory manual", Cold Spring Habor Laboratory, New York (1986)). The microinjection of a corresponding DNA fragment into inseminated mouse oocytes and the subsequent implantation into apparently pregnant females are particularly suited for this purpose. Descendants develop which contain the transgene and pass it on to their descendants (DBD line).

The process according to the invention is carried out in the form of an in vivo assay. Transgenic DBD line animals which are 10 to 15 weeks old are selected and acclimatized correspondingly for the test. 10 to 15 animals (female or male) are required for every treatment. Since an important target organ of the carcinogenesis studies is the skin, the potentially cancerogenic chemical agents are applied topically in each case in 50 to 200 µl solvent, e.g. water, physiological salt solution, acetone or ethanol, two times

a week. For the investigation of potentially carcinogenic physical agents, corresponding applications are also carried out two times a week. These treatments can last up to 20 weeks. In order to enable tumor growth, an additional period of up to 40 to 80 weeks is estimated following the last application. 5 to 50 µg of 7,12-dimethylbenzanthracene (DMBA) can be used in the same test set-up as positive control for the tumor formation. The corresponding solvent which had also been used for dissolving the test substance can be applied as negative control. During the entire test period, the animals are weighed at regular intervals and the site of application is examined. Developing papillomas and other skin tumors, respectively, are investigated macroscopically once a week and optionally measured. As soon as the tumors have reached a critical size (depends on the animal species, the position of the tumor and the national animal protection conditions), the animals are killed and tumor tissue is collected for the histological and molecular-biological characterization. In addition, primary cultures of the tumors can be prepared. The results of the carcinogenicity experiments are evaluated statistically. In this connection, it is possible to determine dose-effect relationships. For the further analysis, it is possible to determine differences in the tumor formation in the sexes and with respect to wild-type mice. Further details on in vivo carcinogenicity assays are found in Tennant et al., Environ. Health Perspect. 103, pp. 942-950 (1995).

As compared to all in vitro models which select individual processes of tumor formation (e.g. DNA damage, mutation production), an in vivo assay whose biological end is the tumor formation as such is more significant. As compared to the above described direct carcinogenicity model using rodents, a sensitivity gain can be obtained by the process according to the invention by using the transgenic mammal wit DNA repair disturbance caused by trans-dominant inhibition of PARP activity so as to reduce the problem of overdosage and production of false positive results. In contrast to the known transgenic mouse models, the process of the present invention bypasses the problem of preparation towards a given tumor formation. The PARP inhibition results in a fundamental DNA repair disturbance and, as a consequence, an increase in the genetic instability (mutation,

recombination, gene amplification rates) following a cancerogenic treatment, so that the tumor formation is then promoted in various ways.

The invention is further described by means of the figures.

Figure 1 shows the expression fragment from pKDinoDBD
K14-prom. = promoter of the human cytokeratin-14 gene
DBD = coding sequence of the DNA binding domain of human poly(ADP ribose)polymerase (EC 2.4.2.30)
p-A = polyadenylation signal of the human cytokeratin-14 gene.

The invention is described in more detail by means of the below examples.

EXAMPLE 1: Production of the transgenic mouse line DBD # 354

The plasmid pKDinoDBD (see figure 1) was cleaved by the restriction enzyme NotI. Following the separation of the restriction fragments on a 1 % agarose gel, a 3.6 kb long fragment which contained the expression cassette of pKDinoDBD, was isolated and prepared by means of a commercially available kit (e.g. "Gene Clean"(r); Dianova company, Hamburg, Germany) according to the manufacturer's instructions. This fragment was adjusted to a concentration of 2 ng/μl in 10 mM Tris-HCl (pH 7.6), 0.25 mM EDTA. F1 females from the crossing of mouse strains C57BL/6 x DBA2 were subjected to superovulation by giving them hormones. After the pairing with F1 males (also by crossing of C57BL/6 x DBA2), inseminated egg cells from the females were prepared and the above described DNA fragment was microinjected thereinto. The embryos were implanted into the uterine tubes of apparently pregnant NMRI mice (nurse mothers; previously paired with vasectomized males). The animals born after about 21 days were tested by means of DNA material from tail biopsies for the presence of the transgene. For this purpose, the technique of polymerase chain reaction (PCR) was used with the following primers from the coding sequence of human PARP (Cherney et al., Proc. Natl. Acad. Sci. USA 84, pp. 8370-8374 (1987)):

5'-ATG GCG GAG TCT TCG GAT AAG CTC TA-3' (primer 1, # 1-26)
5'-GCC AGG CGT GGC CGC CAC GGA GG-3' (primer 2, # 1110-1088)

22 PCR cycles were carried out with 200 ng genomic DNA each. In each case, denaturation was carried out at 95°C for 300 seconds, attachment was made at 60°C for 60 seconds and polymerization took place at 72°C for 120 seconds.

A positive female (earmark #354) was identified. Protein material was obtained from the tail biopsy of this animal and investigated for expression of the transgene by means of Western blot. In this connection, both the monoclonal anti-DBD antibody Cl110 (Lamarre et al., Biochim. Biophys. Acta 950, pp. 147-160 (1988)) and the anti-Fl1 rabbit serum directed against DBD (Küpper et al., J. Biol. Chem. 265, pp. 18721-18724 (1990)) were used. The DNA binding domain (DBD) of 45 kDa could be detected in the Western blot by means of both antibodies, so that the evidence for the expression of the transgene was furnished. The founder-DBD mouse #354 was paired with DBA2 males and the descendants were analyzed. The transgene is passed on to the descendants, so that the line DBD #354 is stably present.

EXAMPLE 2: Identification of the cancerogenic potential of five different chemicals

12-week-old animals of the mouse DBD line #354 described in Example 1 are acclimatized to the test site for three weeks. Female animals are kept in groups of 5 animals per cage, male animals are kept singly under specific pathogen-free (SPF) conditions. The animals are fed according to standard (#D10010 food from Research Diets, New Brunswick, New Jersey, U.S.A., and water ad libitum). 10 to 15 animals (male or female) are required for every treatment. The putatively carcinogenic chemicals to be tested are taken up in several dilution stages in physiological salt solution and acetone, respectively, and 100 µl of every dilution is applied topically in each case two times a week. 20 µg of 7,12-dimethylbenzanthracene (DMBA) in 100 µl acetone are used as positive control. Acetone is applied as negative control. The treatment is carried out for 15 weeks. The animals are weighed

weekly and the application sites are examined. 12 weeks after the end of the treatments, visible tumor growth can be expected in the group of positive control, the tumors rapidly (within 12 further weeks) increasing in size according to experience. Having reached a critical tumor size, the animals are killed by cervical dislocation each and the tumors are removed. As expected, no tumor growth is found in the group of animals treated with solvent even after 60 weeks.

As an alternative, what is called an initiation promotion protocol can be used. In principle, a very low dose of an initiating (usually DNA damaging) carcinogen is administered once in this case, followed by repeated applications of a tumor promoter which is not carcinogenic as such (Becker et al., Cancer Res. 56, pp. 3244-3249, 1996). Here, e.g. nitrosomethylurea (20 µmol in 100 µl acetone; applied topically once) is used as positive control. Seven days later, the treatment is continued with the tumor promoter tetradecanoyl-phorbol-acetate (TPA) two times a week for a period of 22 weeks (in each case 10 mmol in 100 µl acetone). Here, the negative controls are animals which in place of nitrosomethylurea were only given acetone, but then followed by the common TPA treatment. The chemicals to be tested for carcinogenicity are also applied in place of the nitrosomethylurea, again followed by the common TPA treatment. In the case of this protocol, visible tumor growth in the DHD line #354 mouse described in Example 1 has to be expected in the positive control after 9 weeks at the latest.